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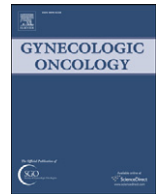
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Status of cellular immunity lacks prognostic significance in vulvar squamous carcinoma

R.A. de Jong^a, N.L. Toppen^a, K.A. ten Hoor^a, H.M. Boezen^b, I.P. Kema^c, H. Hollema^d, H.W. Nijman^{a,*}

^a Department of Gynecologic Oncology, University Medical Center Groningen, University of Groningen, P.O. Box 30.001, 9700 RB Groningen, The Netherlands

^b Department of Epidemiology, University Medical Center Groningen, University of Groningen, P.O. Box 30.001, 9700 RB Groningen, The Netherlands

^c Department of Laboratory Medicine, University Medical Center Groningen, University of Groningen, P.O. Box 30.001, 9700 RB Groningen, The Netherlands

^d Department of Pathology, University Medical Center Groningen, University of Groningen, P.O. Box 30.001, 9700 RB Groningen, The Netherlands

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ABSTRACT

Objective. It is generally recognized that the immune system has an important role in regulating cancer development. Evidence indicating a prognostic role of the immune system in vulvar carcinoma is scarce. This study investigated the presence and prognostic significance of several aspects of the immune system in vulvar squamous carcinoma.

Methods. The number of intratumoral CD8⁺ and Foxp3⁺ T-lymphocytes, next to HLA class I (HLA-A, HLA-B/C and β_2 -m) and indoleamine 2,3-dioxygenase (IDO) expression was determined by immunohistochemistry in a consecutively selected cohort of 286 vulvar squamous carcinoma patients, all treated in the University Medical Center Groningen, the Netherlands. Associations between immunohistochemistry expression and the influence on survival were determined.

Results. The number of tumor-infiltrating CD8⁺ T-lymphocytes was significantly lower in tumors with loss of HLA-A ($p = 0.004$), HLA-B/C ($p = 0.024$) or β_2 -m ($p = 0.025$) expression compared with tumors with expression of HLA class I. No association was found between the number of intratumoral CD8⁺ T-lymphocytes and Foxp3⁺ T-lymphocytes, HLA class I and IDO expression and survival of vulvar squamous carcinoma patients.

Conclusion. Our results indicate that the immune system does not seem to have a major influence on prognosis of patients with vulvar squamous carcinoma.

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Introduction

Vulvar carcinoma is a rare disease accounting for only 5% of all gynecological malignancies [1]. Mainly elderly women are affected with a peak incidence in the eight decade [2]. Squamous cell carcinoma is the most common histological subtype and occurs in 90% of all vulvar carcinomas [3,4]. Stage, tumor size, depth of invasion, vascular space invasion and especially lymph node metastases are important prognostic factors [3]. The 5-year survival is generally good for FIGO stage I (79%) but declines with more advanced stage of disease: stage II (59%), stage III (43%) and stage IV (13%) [2]. Surgery is the cornerstone of vulvar carcinoma treatment and consists of a wide local excision with uni- or bilateral inguinofemoral lymphadenectomy via separate incisions. An important improvement in vulvar cancer treatment was the introduction of sentinel node dissection in selected patients with early stage disease [5]. Adjuvant radiotherapy is indicated in patients with lymph node metastases [6], and chemoradiation is a possibility for (locally) advanced vulvar carcinoma [7].

Despite developments during the last years, improvement of treatment and prognosis of vulvar carcinoma patients is still needed. In this respect, immunotherapy might be an interesting new treatment option where it is generally recognized that the immune system has an important role in regulating cancer development [8].

Cytotoxic T-lymphocytes (CTL) are important effector cells in the adaptive immune system, able to recognize cancer cells as “non-self” and subsequently kill them. Recognition of “non-self” by CTLs can only take place when antigenic peptides (which are derived from digested proteins and broken down in the proteasome of the cell and transported to the cell surface) are presented by the human leukocyte antigen (HLA) class I present at the cell surface of every nucleated cell. The importance of CTL as part of anti-tumor response was emphasized by studies in several cancers demonstrating an association between a high number of tumor-infiltrating CD8⁺ T-lymphocytes (CTL) and increased survival [9–14].

However, cancer cells can develop several strategies to escape eradication by the immune system [15]. Downregulation of the HLA class I molecule is one such powerful mechanism, preventing immune recognition and lysis by CTLs. Another mechanism is the induction of regulatory T-lymphocytes (Tregs) which are known for their immunosuppressive function. Increasing evidence shows that Tregs play a major

* Corresponding author. Fax: +31 503611806.

E-mail address: h.w.nijman@umcg.nl (H.W. Nijman).

role in modulating host response to tumors; by suppressing effector T-lymphocyte proliferation (i.e. CTL) an adequate tumor-specific immune response is prohibited. Increased numbers of intratumoral Tregs was associated with impaired prognosis in several malignancies [16–20]. Furthermore, the intracellular enzyme indoleamine 2,3-dioxygenase (IDO) is currently considered an important immune escape mechanisms in cancer. IDO catalyses the first and rate-limiting steps in the breakdown of the essential amino acid tryptophan along the kynurenine pathway where several downstream metabolites are formed [21]. IDO expression can occur in several cell types (e.g. dendritic cells or cancer cells) after induction by IFN- γ or lipopolysaccharide. IDO exerts its immunosuppressive function by suppressing effector T-lymphocytes and natural killer (NK) cells. Our group recently showed that IDO activity is significantly increased in serum of patients with endometrial, ovarian and vulvar cancer compared to healthy controls [22].

In vulvar carcinoma, evidence for the (prognostic) role of the immune system in vulvar carcinoma is scarce [23,24] and research is warranted on this subject. Therefore, this study aimed to determine the prognostic role of several aspects of the immune system in vulvar carcinoma patients. Therefore, the presence and prognostic influence of two important subsets of T-lymphocytes (CD8⁺ and Foxp3⁺ T-lymphocytes), next to HLA class I downregulation and IDO expression were determined in a large and consecutively selected cohort of patients with vulvar carcinoma.

Materials and methods

Patients

Tissue samples of all patients with gynecological malignancies treated at the Department of Gynecological Oncology of the University Medical Center Groningen (UMCG) are prospectively collected and stored in the tissue storage system of the Department of Pathology of the UMCG, the Netherlands. Clinicopathological characteristics and follow-up data of all these patients are prospectively collected during standard treatment and follow-up and stored in a computerized registration database. For the present study, tissue material and data of clinicopathological characteristics of patients with vulvar carcinoma were used as reported previously [25]. Patients had been consecutively selected when treated for squamous cell carcinoma of the vulva in the UMCG between 1984 and 2001. Patients were excluded when treated with a histological subtype other than squamous cell carcinoma or if they had been treated with preoperative radiotherapy. Staging was performed according to the surgicopathological FIGO classifications applicable at that time [26] and the AJCC TNM classification [27]. Follow-up data were updated until July 2010.

Institutional review board approval

All relevant data were retrieved from our computerized database into a separate, anonymous, password protected database. Patient identity was protected by study specific, unique patient codes, which were only known to two dedicated data managers, who also have responsibility for the larger database. In case of uncertainties with respect to clinicopathological and follow-up data, the larger databases could only be checked through the data managers, thereby ascertaining the protection of patients' identity. Due to these procedures, according to Dutch law no further patient or institutional review board approval was needed.

Immunohistochemistry

For staining, 4- μ m sections were cut from previously constructed tissue microarrays of formalin-fixed paraffin-embedded tumors [25] and applied to 3-amino-propyl-triethoxy-silane coated glass slides (Sigma-Aldrich, Diesenhofen, Germany). For immunostaining, tissue microarray slides were first dewaxed and rehydrated. Methods and

antibodies for immunostaining of CD8⁺ T-lymphocytes, Foxp3⁺ T-lymphocytes, HLA-B/C and β_2 m were used as previously reported [22,28]. For HLA-A and IDO, antigen retrieval was performed by microwave treatment; 15 min in citrate (pH 6.0) (HLA-A), and EDTA buffer (pH 8.0) (IDO). Endogenous peroxidase activity was blocked with 0.3% H₂O₂ for 30 min after which the slides were incubated with the primary antibodies for 60 min at room temperature (dilutions 1:100); HCA2 recognizing the HLA class I heavy chain HLA-A (kindly provided by Prof., Dr. J.J. Neefjes, The Netherlands Cancer Institute, Amsterdam, The Netherlands), and a mouse monoclonal antibody recognizing IDO (anti-indoleamine 2,3-dioxygenase, clone 10.1; Millipore (Chemicon)) were used. Sections were incubated with RAM^{po} (rabbit anti-mouse peroxidase-labeled) and GAR^{po} (goat anti-rabbit peroxidase-labeled) (DAKO, Heverlee, Belgium, 1:100). Antigen-antibody reactions were visualized with 3,3-diaminobenzidine and sections were counterstained with haematoxylin.

Evaluation of immunohistochemistry staining

Immunohistochemistry stained slides were scored independently by two investigators (RJ and NT) when at least 2 cores per case were present, each containing at least 20% tumor tissue. The number of positively stained intratumoral CD8⁺ and Foxp3⁺ T-lymphocytes was counted and the average number was calculated per 0.283 mm² of tumor (i.e. one whole core consisting of 100% tumor tissue) (Figs. 1A and B) [9,10].

IDO expression was assessed according to a semi-quantitative scale which is based on the extent and intensity of the staining [29]. Intensity of the cytoplasmic staining was scored as 0 for absent, 1 for weak, 2 for positive or 3 for strong positive expression. The percentage was scored as 0 for 0–5%; 1 for ≥ 5 –30%; 2 for ≥ 30 –70% and 3 for ≥ 70 –100%. The sum of scores was used to identify four categories of expression: IDO[–] (sum: 0–1), IDO¹⁺ (sum: 2–3), IDO²⁺ (sum: 4–5) and IDO³⁺ (sum: 6) (Figs. 1C and 1D). Mean result of the cores was considered as definitive IDO expression.

A semiquantitative scoring system was also used for HC-10, HC-A2 and β_2 -m expression. Intensity of the membrane staining was scored as 0 for absent; 1 for weak; 2 for positive or 3 for strong positive expression. The percentage was scored as 0 for 0%; 1 for ≥ 1 –5%; 2 for ≥ 5 –25%; 3 for ≥ 25 –50%; 4 for ≥ 50 –75% and 5 for ≥ 75 –100%. The sum of both scores was used to identify three categories of expression: normal expression (6.5–8), partial loss (2.5–6.5) and total loss (0–2.5) (Figs. 1E–M). Mean result of the cores was considered as definitive HC-10, HC-A2 and β_2 -m expression.

Statistical analysis

All continuous variables were tested for normality. In case of skewed distributions, the median and interquartile ranges (IQR, 25th to 75th percentile) were presented. Staining of HLA class I was dichotomized; partial loss and normal expression were taken together and compared to total loss of expression. Associations between clinicopathological characteristics (see Table 1) and the number of CD8⁺ T-lymphocytes, Foxp3⁺ T-lymphocytes (dichotomized according to their median), IDO, HLA-A, HLA-B/C and β_2 -m expression was estimated using univariate logistic regression analyses. Clinicopathological parameters were used as independent and T-lymphocytes, IDO expression and HLA class I components were used as dependent variables. Odds Ratios (ORs) and 95%-confidence intervals (95%-CI) were estimated. Univariate and multivariate Cox proportional hazard analysis were used to assess influence of clinicopathological characteristics, numbers of CD8⁺ T-lymphocytes and Foxp3⁺ T-lymphocytes and expression levels of IDO, HLA-A, HLA-B/C and β_2 -m on survival. Disease-free survival (DFS) was defined as date of diagnosis until recurrent disease, metastasis, death due to vulvar cancer or last date of follow-up. Disease specific survival (DSS) was defined as date of diagnosis until death due to vulvar

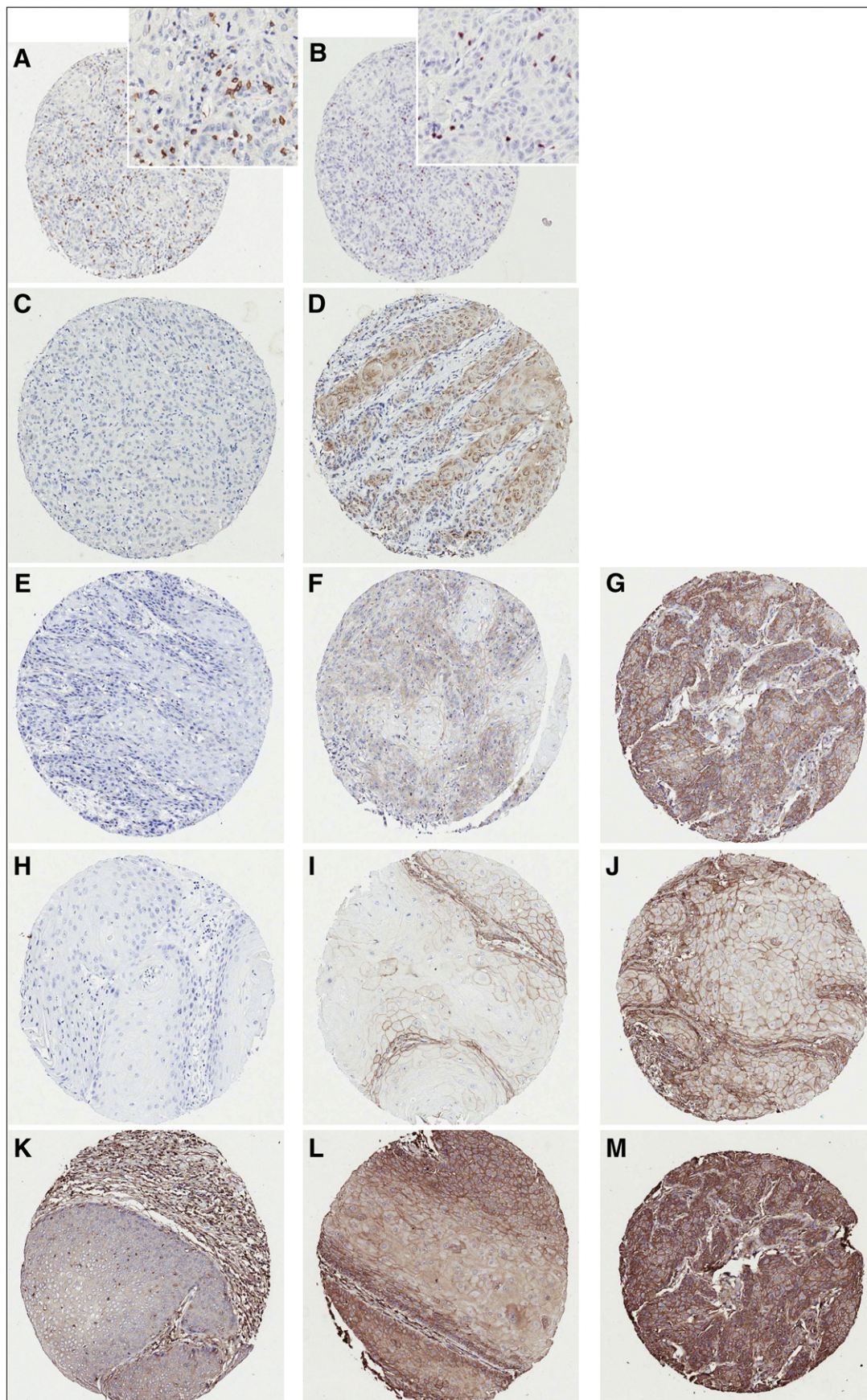


Fig. 1. Immunohistochemistry staining of intratumoral CD8⁺ T-lymphocytes (A) and Foxp3⁺ T-lymphocytes (B), IDO expression ; negative (C), positive (D) and HLA class I heavy chain HCA2 (E–G), HC10 (H–J) and β₂-m (K–M) (loss of expression: E,H,K; partial loss: F, I, L; normal expression: G, J, M).

Table 1
Clinicopathological characteristics of 286 patients with vulvar carcinoma.

	Patients (n = 286) (%) ^a
<i>Age (years)</i>	
Median (IQR)	73 (63–81)
<i>Differentiation grade</i>	
Good	112 (39.3)
Moderate	129 (45.3)
Poor	44 (15.4)
Missing	1
<i>T-status</i>	
T1 (maximum diameter ≤ 2 cm)	74 (25.9)
T2 (maximum diameter > 2 cm)	212 (74.1)
Missing	0
<i>Infiltration depth</i>	
≤ 5 mm	116 (41.3)
> 5 mm	165 (58.7)
Missing	5
<i>Vascular invasion</i>	
Negative	220 (82.7)
Positive	46 (17.3)
Missing	20
<i>Tumor localisation</i>	
Unifocal	241 (84.3)
Multifocal	45 (15.7)
Missing	0
<i>Inguinofemoral metastasis</i>	
No metastasis	157 (59.9)
Metastasis present	105 (40.1)
Missing	24
<i>Radiotherapy</i>	
No	192 (67.1)
Yes	94 (32.9)
Missing	0
<i>Recurrence of disease</i>	
No recurrence	173 (64.1)
Recurrence	97 (35.9)
Locoregional	82 (84.5)
Distant	15 (15.5)
Missing	17

^a Percentages exclude missing cases.

carcinoma or the date of last follow-up. Clinicopathological characteristics and immunohistochemistry expression were used as independent and recurrent disease (DFS) or death of disease (DSS) were used as dependent variables. Hazard Ratios (HRs) and 95%-CIs were calculated. Variables with a *p* value < 0.05 in univariate analysis were simultaneously entered into the multivariate model. All tests were performed two-sided and *p* values of < 0.05 were considered statistically significant. Analyses were performed using SPSS software version 16.0 (SPSS Inc, Chicago, USA).

Results

Patients

In total, 286 patients with vulvar carcinoma were included in this study of whom clinicopathological characteristics are depicted in Table 1. Median age at time of diagnosis was 73 years (IQR: 63–81 years). The majority of the patients were diagnosed with a T2 tumor (74.1%). The tumor was localized unilateral in 84.3% and multifocality was observed in 15.7%. One or more inguinofemoral metastasis were present in 40.1% of the patients whereas 59.9% did not show inguinofemoral metastasis.

Associations between clinicopathological characteristics and immunological factors

CD8⁺ and Foxp3⁺ T-lymphocytes

Intratumoral CD8⁺ and Foxp3⁺ T-lymphocytes were present in 96.3% (median 36; IQR 14–93) and 95.1% (median 17; IQR 7–42). The median CD8⁺/Foxp3⁺ ratio was 1.73 (IQR 0.97–3.86). Tumors with infiltration depth of > 5 mm were associated with a high number of CD8⁺ T-lymphocytes and a high CD8⁺/Foxp3⁺ ratio (Table 2). A T2 tumor more frequently had a high CD8⁺/Foxp3⁺ T-lymphocytes ratio. No associations were found between clinicopathological characteristics and the number of Foxp3⁺ T-lymphocytes.

HLA class I downregulation

Downregulation of HLA-A, HLA-B/C or β₂-m was observed in 37.9%, 11.1% and 2.2%, respectively. Age and positive vascular invasion were associated with HLA-A downregulation and poorly differentiated tumors were associated with HLA-B/C downregulation (Table 3). A low number of CD8⁺ T-lymphocytes was associated with downregulation of HLA-A, HLA-B/C and β₂-m. Furthermore, a high number of Foxp3⁺ T-lymphocytes was associated with downregulation of HLA-A, HLA-B/C and β₂-m.

IDO expression

IDO expression was present in 50.4% of the cases. As shown by means of univariate logistic regression analysis (Table 3), no associations were found between clinicopathological characteristics, HLA class I downregulation, the number of T-lymphocytes and IDO expression.

Survival

Median time of follow-up was 73 months (IQR: 0–290 months). In 98 patients (32.6%), recurrence of disease was diagnosed with a median time until recurrence of 23 months (IQR: 7.0–65.0 months). In total, 76 patients (25.5%) died as a result of vulvar carcinoma during our follow-up.

In univariate analyses on DFS, only the presence of inguinofemoral metastasis was significantly related to a shorter DFS (Table 4).

In univariate analyses on DSS, inguinofemoral metastasis, T2 tumor, a high tumor grade, positive vascular invasion and infiltration depth > 5 mm were predictors for a worse DSS, in order of importance (Table 5). In multivariate analysis, inguinofemoral metastasis, T2 tumor, a high tumor grade and positive vascular invasion were independent predictors for a shorter DSS.

The number of CD8⁺ T-lymphocytes and Foxp3⁺ T-lymphocytes, HLA class I downregulation, IDO expression were not related to DSS.

Discussion

In this large and well-documented cohort of 286 patients with vulvar carcinoma, we evaluated the prognostic significance of several aspects of the immune system. We showed that the number of CD8⁺ T-lymphocytes was significantly related to HLA class I downregulation which reflects the natural mechanism of this interaction. However, the number of intratumoral CD8⁺ T-lymphocytes or Foxp3⁺ T-lymphocytes, HLA class I downregulation and IDO expression were not associated with survival. Our results indicate that the immune system does not seem to have a major influence on prognosis of patients with vulvar carcinoma.

The prognostic influence of tumor-infiltrating T-lymphocytes and more specifically, CD8⁺ and Foxp3⁺ T-lymphocytes has been studied in several cancer types. In this cohort of 286 vulvar carcinoma patients, intra-tumoral CD8⁺ and Foxp3⁺ T-lymphocytes were present in 96.3% and 95.1% of the cases. In agreement with results of a recently published study in vulvar carcinoma [23], we did not find an association between the number of T-lymphocytes and survival of vulvar

Table 2Association between CD8⁺ T-lymphocytes, Foxp3⁺ T-lymphocytes, CD8⁺/Foxp3⁺ ratio and clinicopathological factors in 286 vulvar carcinoma patients.

	CD8 ⁺ T-lymphocytes		Low CD8 ⁺ T-lymphocytes	p value
	<Median	≥Median	OR (95% CI)	
Age			1.00 (0.98–1.02)	0.831
Tumor grade 3/undifferentiated	16/104 (15.4%)	14/114 (12.2%)	1.30 (0.60–2.81)	0.507
T-status: T2 (max. diameter > 2 cm)	75/105 (71.4%)	84/114 (73.7%)	0.89 (0.49–1.62)	0.709
Infiltration depth > 5 mm	53/102 (52.0%)	74/113 (65.5%)	0.57 (0.33–0.99)	0.045
Vascular invasion positive	17/102 (16.7%)	18/103 (17.5%)	0.94 (0.46–1.96)	0.878
Multifocality	14/105 (13.3%)	24/114 (21.1%)	0.58 (0.28–1.19)	0.135
Inguinofemoral metastasis	35/94 (37.2%)	44/106 (41.5%)	0.84 (0.47–1.48)	0.537
	Foxp3 ⁺ T-lymphocytes		High Foxp3 ⁺ T-lymphocytes	p value
	<Median	≥Median	OR (95% CI)	
Age			0.99 (0.97–1.01)	0.440
Tumor grade 3/undifferentiated	13/99 (13.1%)	20/123 (16.3%)	1.29 (0.60–2.73)	0.515
T-status: T2 (max. diameter > 2 cm)	78/100 (78.0%)	84/123 (68.3%)	0.61 (0.33–1.11)	0.107
Infiltration depth > 5 mm	58/96 (60.4%)	72/123 (58.5%)	0.93 (0.54–1.59)	0.779
Vascular invasion positive	12/89 (13.5%)	24/119 (20.2%)	1.62 (0.76–3.45)	0.210
Multifocality	14/100 (14.0%)	25/123 (20.3%)	1.57 (0.77–3.21)	0.219
Inguinofemoral metastasis	33/89 (37.1%)	49/114 (43.0%)	1.28 (0.73–2.26)	0.395
	CD8 ⁺ /Foxp3 ⁺ ratio		Low CD8 ⁺ /Foxp3 ⁺ ratio	p value
	<Median	≥Median	OR (95% CI)	
Age			1.00 (0.98–1.02)	0.715
Tumor grade 3/undifferentiated	16/106 (13.8%)	14/107 (13.1%)	1.18 (0.55–2.56)	0.673
T-status: T2 (max. diameter > 2 cm)	70/107 (65.4%)	86/107 (80.4%)	0.46 (0.25–0.86)	0.015
Infiltration depth > 5 mm	53/105 (50.5%)	73/105 (69.5%)	0.45 (0.25–0.79)	0.005
Vascular invasion positive	22/105 (21.0%)	13/95 (13.7%)	1.67 (0.79–3.54)	0.180
Multifocality	20/107 (18.7%)	17/107 (15.9%)	1.22 (0.60–2.48)	0.588
Inguinofemoral metastasis	37/95 (38.9%)	41/100 (41.0%)	0.92 (0.52–1.63)	0.770

Bold values signify $p < 0.05$.

carcinoma patients. This result is in contrast with studies previously performed in endometrial [9,11], ovarian [10,30] and cervical carcinoma [31].

In this study, vulvar carcinomas with infiltration depth of > 5 mm were associated with a high number of CD8⁺ T-lymphocytes and a high CD8⁺/Foxp3⁺ ratio. Furthermore, a T2 tumor more frequently had a high CD8⁺/Foxp3⁺ T-lymphocytes ratio. These results are in contrast to results found in other cancer types where high numbers of CD8⁺ and low numbers of Foxp3⁺ T-lymphocytes were associated with favourable outcome [9–12,32]. The fact that larger tumors are associated with infiltration with more T-lymphocytes suggests that infiltration did not result in effective eradication of the tumor. A possible cause might be the simultaneous downregulation of HLA class I, which prevents recognition and lysis of the cancer cells by CD8⁺ T-lymphocytes. This hypothesis is supported by results of univariate logistic regression analyses; the number of tumor-infiltrating CD8⁺ T-lymphocytes was significantly lower in tumors with downregulation of HLA class I (either loss of HLA-A, HLA-B/C or β_2 -m expression) compared with tumors with expression of HLA class I.

Younger patients with vulvar carcinoma more frequently showed downregulation of HLA-A. Furthermore, positive vascular invasion was associated with HLA-A downregulation, and poorly differentiated tumors were associated with HLA-B/C downregulation. These results suggest an association between poor prognostic factors and downregulation of HLA class I. However, HLA class I downregulation did not have a significant influence on survival of patients with vulvar carcinoma. These results are in contrast to studies in endometrial [28], ovarian [33,34] and cervical cancer [31,35] where an association between downregulation of HLA class I and poor prognosis was observed.

IDO is currently considered an important immune escape mechanism in cancer. We have recently shown that IDO activity is significantly increased in serum of patients with vulvar cancer [22], suggesting that IDO also plays a role in vulvar carcinoma. IDO expression has been associated with decreased prognosis in several cancer

types. In contrast to results of a recent study in 76 vulvar carcinoma patients [24], we did not find an association between IDO expression and clinicopathological characteristics or survival. The intracellular enzyme IDO functions as an immune escape mechanism by means of suppressing T-lymphocytes and NK cells and therefore we expected an inverse relation between the number of CD8⁺ T-lymphocytes and IDO expression. However, we did not observe such a relation. These results suggest that the immune system has a different function in vulvar carcinoma compared to other gynecological malignancies such as endometrial, ovarian and cervical cancer. Possibly, the fact that vulvar carcinoma is localized on the skin might cause these differences. One other explanation might be the fact that patients diagnosed with vulvar carcinoma are generally older compared to patients diagnosed with endometrial, ovarian or cervical cancer. Aging is associated with a change in immune activation, especially of the T-cell/macrophage system. Due to an IFN- γ dependent increase of IDO activity amongst elderly, infections, autoimmune diseases and malignancies occur more often [36].

The presence of inguinofemoral metastasis was the only factor which significantly related to a shorter DFS. Furthermore, the presence of inguinofemoral metastasis was an independent predictor for a worse DSS, next to a T2 tumor, poorly/undifferentiated differentiated tumor and positive vascular invasion. These results are in agreement with previous studies determining the prognostic role of several surgicopathological characteristics in patients with vulvar carcinoma [3].

This study evaluated the presence and prognostic influence of important players in cellular immunity and to our knowledge, we are the first to evaluate HLA class I downregulation in vulvar carcinoma. We were able to evaluate immunohistochemistry expression on tissue material of a unique large number ($n = 286$) of vulvar carcinoma patients, all treated with primary surgery in a single institution (UMCG, Groningen, the Netherlands). In order to evaluate this large series of specimens in a high-throughput manner, we used a

Table 3

Association between clinicopathological characteristics and HLA class I downregulation and IDO expression in 286 vulvar cancer patients.

	HLA-A		HLA-A downregulation	p value
	Loss of expression (n = 86)	Expression (n = 141)	OR (95% CI)	
Age			0.98 (0.96–1.00)	0.027
Tumor grade 3/undifferentiated	15/86 (17.4%)	19/140 (13.6%)	1.35 (0.64–2.81)	0.430
T-status: T2 (max. diameter > 2 cm)	65/86 (75.6%)	101/141 (71.6%)	1.23 (0.66–2.26)	0.515
Infiltration depth > 5 mm	35/85 (41.2%)	78/138 (56.5%)	1.27 (0.73–2.22)	0.391
Vascular invasion positive	20/82 (24.4%)	17/130 (13.1%)	2.14 (1.05–4.39)	0.037
Multifocality	11/86 (12.8%)	30/141 (21.3%)	0.54 (0.26–1.15)	0.110
Inguinofemoral metastasis	35/78 (44.9%)	48/131 (36.6%)	1.41 (0.80–2.49)	0.240
IDO expression	45/84 (53.6%)	66/133 (49.6%)	1.17 (0.68–2.02)	0.571
CD8 ⁺ T-lymphocytes			1.00 (0.99–1.00)	0.080
CD8 ⁺ T-lymphocytes < median	52/85 (61.2%)	53/129 (41.1%)	2.26 (1.29–3.96)	0.004
Foxp3 ⁺ T-lymphocytes			0.98 (0.97–0.99)	0.002
Foxp3 ⁺ T-lymphocytes ≥ median	33/84 (39.3%)	85/133 (63.9%)	2.74 (1.56–4.81)	<0.001
CD8 ⁺ /Foxp3 ⁺ ratio			1.01 (0.99–1.02)	0.373
	HLA-B/C		HLA-B/C downregulation	p value
	Loss of expression (n = 25)	Expression (n = 201)	OR (95% CI)	
Age			1.01 (0.98–1.05)	0.490
Tumor grade 3/undifferentiated	9/27 (33.3%)	23/200 (11.5%)	4.33 (1.72–10.92)	0.002
T-status: T2 (max. diameter > 2 cm)	20/27 (74.1%)	148/201 (73.6%)	0.92 (0.36–2.33)	0.862
Infiltration depth > 5 mm	20/27 (74.1%)	114/197 (57.9%)	1.87 (0.75–4.69)	0.180
Vascular invasion positive	7/26 (26.9%)	30/187 (16.0%)	2.16 (0.82–5.65)	0.118
Multifocality	4/27 (14.8%)	37/201 (18.4%)	0.84 (0.27–2.61)	0.768
Inguinofemoral metastasis	11/23 (47.8%)	71/184 (38.6%)	1.33 (0.55–3.23)	0.534
IDO expression	15/25 (60.0%)	96/194 (49.5%)	1.91 (0.78–4.72)	0.159
CD8 ⁺ T-lymphocytes			0.99 (0.99–1.00)	0.159
CD8 ⁺ T-lymphocytes < median	17/25 (68.0%)	86/189 (45.5%)	2.91 (1.13–7.34)	0.024
Foxp3 ⁺ T-lymphocytes			0.94 (0.91–0.98)	0.002
Foxp3 ⁺ T-lymphocytes ≥ median	4/25 (16.0%)	115/193 (59.6%)	0.14 (0.05–0.41)	<0.001
CD8 ⁺ /Foxp3 ⁺ ratio			1.00 (0.99–1.02)	0.613
	β ₂ -m		β ₂ -m downregulation	p value ^a
	Loss of expression (n = 5)	Expression (n = 221)	OR (95% CI)	
Age				0.663
Tumor grade 3/undifferentiated	0/5 (0%)	34/220 (15.5%)	n.a.	0.340
T-status: T2 (max. diameter > 2 cm)	5/5 (100%)	162/221 (73.3%)	n.a.	0.330
Infiltration depth > 5 mm	3/5 (60%)	127/216 (58.8%)	n.a.	0.957
Vascular invasion positive	1/5 (20.0%)	35/205 (17.1%)	n.a.	0.864
Multifocality	1/5 (20.0%)	40/221 (18.1%)	n.a.	0.913
Inguinofemoral metastasis	3/5 (60.0%)	81/203 (39.9%)	n.a.	0.366
IDO expression	2/4 (40.0%)	110/213 (51.6%)	n.a.	0.330
CD8 ⁺ T-lymphocytes			n.a.	n.a.
CD8 ⁺ T-lymphocytes < median	5/5 (100%)	98/208 (47.1%)	n.a.	0.025
Foxp3 ⁺ T-lymphocytes			n.a.	n.a.
Foxp3 ⁺ T-lymphocytes ≥ median	0/5 (0%)	118/212 (55.7%)	n.a.	0.019
CD8 ⁺ /Foxp3 ⁺ ratio			n.a.	0.650
	IDO		IDO high expression	p value
	Negative	Positive	OR (95% CI)	
Age			1.01 (0.99–1.03)	0.341
Tumor grade 3/undifferentiated	12/116 (10.3%)	20/120 (16.7%)	1.65 (0.76–3.58)	0.206
T-status: T2 (max. diameter > 2 cm)	87/117 (74.4%)	87/120 (72.5%)	0.90 (0.50–1.61)	0.717
Infiltration depth > 5 mm	69/113 (61.1%)	70/118 (59.3%)	0.90 (0.53–1.54)	0.707
Vascular invasion positive	16/109 (14.7%)	23/109 (21.1%)	1.44 (0.70–3.00)	0.325
Multifocality	19/117 (16.2%)	20/120 (16.7%)	0.98 (0.49–1.97)	0.953
Inguinofemoral metastasis	39/103 (37.9%)	46/110 (41.8%)	1.21 (0.69–2.11)	0.507
HLA-A downregulation	42/110 (38.2%)	45/115 (39.1%)	1.17 (0.68–2.02)	0.571
HLA-B/C downregulation	10/110 (9.1%)	15/116 (12.9%)	1.91 (0.78–4.72)	0.159
β ₂ -m downregulation	2/109 (1.8%)	2/116 (1.7%)	0.94 (0.13–6.77)	0.948
CD8 ⁺ T-lymphocytes			1.00 (1.00–1.00)	0.936
CD8 ⁺ T-lymphocytes < median	54/110 (49.1%)	56/116 (48.3%)	0.93 (0.55–1.59)	0.796
Foxp3 ⁺ T-lymphocytes			1.00 (0.99–1.01)	0.758
Foxp3 ⁺ T-lymphocytes ≥ median	58/109 (53.2%)	65/116 (56.0%)	1.17 (0.69–1.99)	0.565
CD8 ⁺ /Foxp3 ⁺ ratio			1.01 (0.99–1.02)	0.389

FIGO = International Federation of Gynecology and Obstetrics.

n.a. = not applicable.

^a Statistical analysis by Pearson's Chi-square.

previously constructed tissue microarray [25]. This method is widely used in cancer research and was recently validated for vulvar carcinoma [37]. Our results showed that none of these immunological

parameters have a significant influence on survival of vulvar carcinoma patients, in contrast to results of studies in several other cancer types. However, as there is a relationship between effect and study size, we

Table 4
COX regression analysis on disease free survival.

	Univariate analysis			Multivariate analysis
	HR	95% CI	p value	
Age	1.01	1.00–1.03	0.126	^a
Tumor grade 3/undifferentiated	1.42	0.83–2.43	0.203	^a
T-status: T2 (max. diameter > 2 cm)	1.38	0.87–2.19	0.171	^a
Infiltration depth > 5 mm	1.30	0.86–1.95	0.217	^a
Vascular invasion positive	1.60	0.94–2.72	0.082	^a
Multifocality	1.48	0.90–2.42	0.119	^a
Inguinofemoral metastasis	2.23	1.48–3.36	<0.001	^a
IDO expression	1.24	0.79–1.93	0.348	^a
HLA-A downregulation	0.95	0.59–1.51	0.813	^a
HLA-B/C downregulation	0.76	0.33–1.76	0.526	^a
β_2 -m downregulation	0.55	0.08–3.94	0.550	^a
CD8 ⁺ T-lymphocytes	1.00	0.99–1.00	0.067	^a
Foxp3 ⁺ T-lymphocytes	1.00	0.99–1.01	0.899	^a
CD8 ⁺ /Foxp3 ⁺ ratio	1.00	0.98–1.01	0.438	^a

Bold values signify $p < 0.05$.

^a Not included in multivariate analysis.

Table 5
COX regression analysis on disease specific survival.

	Univariate analysis			Multivariate analysis		
	HR	95% CI	p value	HR	95% CI	p value
Age	1.01	1.00–1.03	0.235	^a		
Tumor grade 3/undifferentiated	2.79	1.63–4.76	<0.001	1.97	1.10–3.55	0.023
T-status: T2 (max. diameter > 2 cm)	3.89	1.78–8.54	0.001	3.12	1.19–8.17	0.020
Infiltration depth > 5 mm	2.17	1.27–3.70	0.004	0.97	0.52–1.81	0.924
Vascular invasion positive	2.74	1.58–4.77	<0.001	1.94	1.07–3.50	0.028
Multifocality	1.10	0.59–2.05	0.772	^a		
Inguinofemoral metastasis	6.02	3.38–10.72	<0.001	5.30	2.81–10.01	<0.001
IDO expression	1.30	0.73–2.32	0.366	^a		
P16 positive	0.91	0.49–1.69	0.763	^a		
HLA-A downregulation	0.85	0.47–1.55	0.600	^a		
HLA-B/C downregulation	0.98	0.39–2.48	0.970	^a		
β_2 -m downregulation	0.92	0.13–6.68	0.935	^a		
CD8 ⁺ T-lymphocytes	1.00	1.00–1.00	0.726	^a		
Foxp3 ⁺ T-lymphocytes	1.00	1.00–1.01	0.301	^a		
CD8 ⁺ /Foxp3 ⁺ ratio	1.00	0.99–1.01	0.985	^a		

Bold values signify $p < 0.05$.

^a Not included in multivariate analysis.

believe that our (negative) results in a large cohort of patients is of major importance [13].

In summary, the current study investigated the presence and prognostic impact of several immunological factors in vulvar carcinoma patients. The number of tumor-infiltrating CD8⁺ and Foxp3⁺ T-lymphocytes, HLA class I downregulation and IDO expression were not related to survival in our population. The presence of inguinofemoral metastasis was one of the “classical” prognostic characteristics associated with poor survival and is of major importance in determining prognosis and adequate treatment strategies in patients with vulvar carcinoma. Although our results suggest that important players in the adaptive immune system do not seem to have an influence on survival, these results need to be confirmed (or rejected) by future research. Attempts have been made to identify tumor associated antigens as possible targets in vulvar carcinoma [38]. Hopefully, this will lead to more research concerning targeted therapy in an approach to improve prognosis of vulvar carcinoma patients.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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